Exhibit O

The Volume of Saliva in the Mouth Before and After Swallowing

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In 20 male and 20 female adult subjects, the volume of saliva in the mouth before (VMAX) and after (RESID) swallowing was determined. RESID could be computed by measuring the potassium and chloride concentrations in unstimulated saliva and in the expectorate after a five-second rinse with 5 ml of water immediately following a swallow. The mean value of RESID after a normal swallow was significantly higher in males (0.87 ml) than in females (0.66 ml). After a forced swallow, RESID was only slightly but significantly reduced, being 0.82 ml and 0.60 ml in males and females, respectively. The volume of saliva normally swallowed was calculated from the unstimulated salivary flow rate and the normal swallowing frequency. The mean value of VMAX (RESID plus volume normally swallowed) in males was 1.19 ml, which was slightly but not significantly higher than that in females (0.96 ml). When water was infused into the mouth at increasing flow rates, there was an increase in VMAX and in both the volume of fluid swallowed and the swallowing frequency.

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Introduction.

It is well-established that sugar has an important role in the development of dental caries (Newbrun, 1978). The availability of sugar to the dental plaque, the site where acids are formed by bacterial fermentation of carbohydrates, is strongly dependent on the sugar concentration in the surrounding saliva (Kleinberg, 1961; Frostell, 1969). Sugar is eliminated from the mouth by dilution with newly secreted saliva, followed by swallowing – a process generally referred to as sugar clearance.

Recently, a mathematical model of salivary clearance of sugar from the oral cavity has been formulated (Dawes, 1983) which takes into account the swallowing process and the dependency of salivary flow rate on the salivary sugar concentration. The parameters in this model include the amount of sugar in the mouth at time zero, the delay between taste stimulation and development of a steady stimulated flow rate, the taste threshold for sugar, the sugar concentration in a saturated solution, the unstimulated salivary flow rate, the maximum stimulated flow rate, the volume of saliva in the mouth initially, and the volume in the mouth before and immediately after swallowing (VMAX and RESID, respectively).

By computer simulation, the most important of these parameters appear to be the unstimulated salivary flow rate and the volumes of saliva in the mouth immediately before and after swallowing. The first of these parameters has been extensively investigated, but there do not appear to be any data in the literature on the values for the latter two, other than in an abstract by Dodds & Edgar (1983) which suggests that the value of RESID falls into the range of 0.3 - 0.6 ml. Therefore, the purpose of the present study was to determine normal values for VMAX and RESID. Furthermore, since it seemed likely that the volume of saliva in the mouth before swallowing would be dependent on the salivary flow rate, we also determined the volume swallowed when fluid was infused into the mouth at different flow rates.

Materials and methods.

Subjects. – The subjects were 20 males and 20 females who were all in good health and ranged in age from 18 to 47 (mean, 21 years). Their height, weight, DMFT-index (number of decayed, missing, or filled teeth), and DMFS-index (number of decayed, missing, or filled tooth surfaces) were recorded. All the subjects had more than 24 natural teeth, and none wore dentures, bridges, or orthodontic appliances which might have retained saliva.

Determination of RESID. – The volume of saliva left in the mouth after swallowing was measured by a dilution method. After collecting about 0.5 ml of unstimulated whole saliva, the subject swallowed, and immediately afterward about 5 ml of distilled water in a beaker was used to rinse the mouth for five sec prior to expectoration into another container. The volume of water used was determined by the difference in weight of the beaker before and after rinsing. On each subject, five such trials were performed, during which the subject was asked to wait for a normal swallow to occur, and another five trials during which the subject swallowed as forcefully as possible. The trials were separated by intervals of a few minutes.

The saliva and the expectorate were analyzed for potassium by atomic absorption spectrophotometry (Dawes, 1969) and for chloride by a coulometric technique (Cotlove *et al.*, 1958).

If V is the volume of water introduced into the mouth after swallowing, and C_i and C_f are the concentrations of potassium or chloride in the saliva and expectorate, respectively, then:

$$\text{RESID} \cdot C_i = (\text{V} + \text{RESID}) \cdot C_f \text{ and RESID} = \frac{\text{V} \cdot C_f}{C_i - C_f}$$

In this formula it is assumed that salivary secretion during the five sec of rinsing is negligible, and preliminary studies on subjects wearing parotid cannulae confirmed this. Preliminary studies also indicated that the rinse time was not critical. When the saliva and expectorate from one trial were each divided into ten aliquots and each aliquot was analyzed for both potassium and chloride, the mean value of RESID from the ten pairs of analyses was 0.40 ml, with a S.D. of 0.01 ml and a coefficient of variation of 2.5%.

Mixed analysis of variance (ANOVA) showed that the value of RESID obtained from the chloride data exceeded by 6.8% that determined from the potassium analyses ($p < 0.01$). However, this difference represents a clinically insignificant volume difference of about 50 μl, and hence the data for RESID are reported as the mean from the chloride and potassium analyses.

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Determination of the volume swallowed and VMAX. – The volume swallowed was calculated as salivary flow rate divided by the swallowing frequency, and VMAX was estimated as the sum of RESID and the volume of saliva swallowed. The volume swallowed was also determined while fluid was infused into the mouth at different flow rates.

With the subject seated in an upright position, two polyethylene tubes (diameter, 1.5 mm) were placed in the buccal sulcus, terminating opposite the first upper molar teeth. The tubes were connected to peristaltic pumps[1] through which 10 mM NaCl at 37°C, to simulate the composition of unstimulated saliva, was infused at 11 different flow rates which ranged from zero to 62 ml/min, selected in a randomized order. The flow rates varied slightly between individuals because it was not possible to reproduce exactly the same control settings on the pump. However, the actual flow rates in each subject were measured by weighing the fluid exiting from the tubes over a known time.

Swallows were registered by placing over the larynx a strain gauge[2] which was connected to a recorder[3] *via* a cardiac pre-amplifier and a transducer monitor coupler[2]. Initially, the timing of the characteristic patterns on the tracing due to swallowing was confirmed by close observation of the subject. Knowing the flow rate from the pump plus the unstimulated salivary flow rate, and assuming that RESID remained constant, the volume of fluid swallowed at each swallow could be determined for the last 12 swallows on the recorded trace, which for each flow rate continued for up to ten min.

The unstimulated salivary flow rate was determined before and after each session. With the subject seated upright and with the head bent forward, the subject swallowed, and saliva was then allowed to drip off the lower lip into a weighed beaker for five min, at the end of which any remaining saliva in the mouth was expectorated into the beaker, and the increase in weight was determined.

Results.

The Table shows the results for the residual volume after swallowing (RESID) for both normal and forced swallows. Mixed ANOVA revealed that the value of RESID after a normal swallow (0.77 ml) was significantly larger ($p < 0.01$) than that after a forced swallow (0.71 ml), and that the mean value of RESID in males was significantly larger than that in females ($p < 0.001$) for both normal and forced swallows.

For the five estimations of RESID on each subject, the mean coefficient of variation after normal swallows was 13.5%, while after forced swallows it was 12.7%, and there was no significant difference between males and females in this respect.

When the results for the two sexes were analyzed separately, there was no correlation between RESID and height or weight. However, in males, but not females, RESID was significantly correlated with both DMFT ($r = 0.435$, $p = 0.027$) and DMFS ($r = 0.381$, $p = 0.048$).

The unstimulated salivary flow rate averaged 0.49 ± 0.23 (S.D.)ml/min (range 0.14-1.01 ml/min) before the fluid infusion experiments began and 0.51 ± 0.27 (S.D.)ml/min (range 0.11-1.20 ml/min) at the end, a non-significant difference. Although males showed a higher flow rate at both time points (0.53 ± 0.22 ml/min and 0.58 ± 0.27 ml/min) than did females (0.46 ± 0.24 ml/min and 0.43 ± 0.24 ml/min), the differences were not statistically significant.

TABLE
THE VOLUME OF SALIVA (ML) IN THE MOUTH BEFORE (VMAX) AND AFTER (RESID) NORMAL OR FORCED SWALLOWING

	Number of Subjects	Mean ± SD	Range
RESID, normal			
males & females	40	0.77 ± 0.23	0.38-1.73
males	20	0.87 ± 0.25	0.62-1.73
females	20	0.66 ± 0.14	0.38-0.97
RESID, forced			
males & females	40	0.71 ± 0.20	0.37-1.41
males	20	0.82 ± 0.19	0.48-1.41
females	20	0.60 ± 0.13	0.37-0.94
VMAX			
males & females	40	1.07 ± 0.39	0.52-2.14
males	20	1.19 ± 0.39	0.77-2.14
females	20	0.96 ± 0.37	0.52-1.94

At the unstimulated salivary flow rate, the mean volume of the liquid bolus swallowed was 0.30 ± 0.27 (S.D.) ml (range 0.09-1.12 ml), and the mean swallowing frequency was 2.3 ± 1.3 (S.D.) swallows per min. There was no significant difference between males and females in either the volume swallowed or the swallowing frequency.

The volume of saliva in the mouth before swallowing (VMAX) is the sum of RESID plus the volume swallowed, and the results for VMAX in the absence of fluid infusion into the mouth are also shown in the Table. VMAX was larger in males (1.19 ml) than in females (0.96 ml), but the difference was not statistically significant.

When the results for both sexes were analyzed separately, VMAX was not significantly correlated with height, weight, DMFT, or DMFS.

The presence in the buccal sulcus of the two tubes used to infuse fluid did not significantly alter the volume of saliva swallowed at the unstimulated flow rate. However, infusion of fluid increased both the volume swallowed (Fig. 1) and the frequency of swallowing (Fig. 2). Both curves tended to asymptote at high rates of fluid infusion, and the pattern of swallowing varied greatly between subjects, as may be seen from the large standard deviations in Figs. 1 & 2. Fig. 1 shows the volume swallowed for infusion rates up to 10.6 ml/min, which we have found to be about the maximum sugar-stimulated salivary flow rate. However, the data in Fig. 2 allow the volume swallowed to be calculated for higher fluid infusion rates. At an infusion rate of 62 ml/min, the volume swallowed with each swallow was 3.94 ml.

Discussion.

The clearance model of Dawes (1983) is applicable to all soluble or well-dispersed substances introduced into the oral cavity, which are not either bound to or absorbed from its surfaces. The major concern, however, has been with the clearance rate of sucrose, the main substrate for acid formation by the micro-organisms causing dental caries. Computer simulations show that the lower the volumes in the oral cavity before and after swallowing, the faster is the sugar cleared from the mouth, thus resulting in a lower

[1]Masterflex Model 7562-00, Cole Parmer Instrument Co., Chicago, IL

[2]E & M Instrument Co., Houston, TX

[3]Omniscribe, Industrial Scientific, Inc., Houston, TX

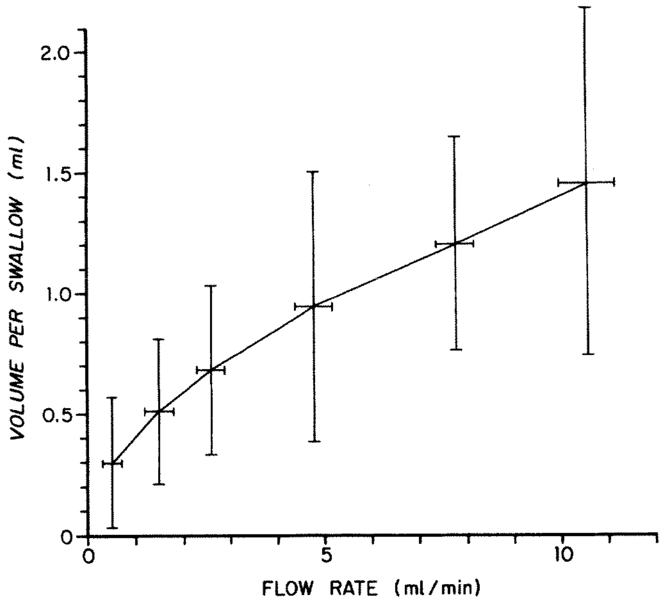

Fig. 1 – The effect of fluid infusion rate on the volume of fluid swallowed with each swallow. The vertical lines indicate standard deviations for the volume swallowed, while the horizontal lines indicate standard deviations for the fluid flow rate.

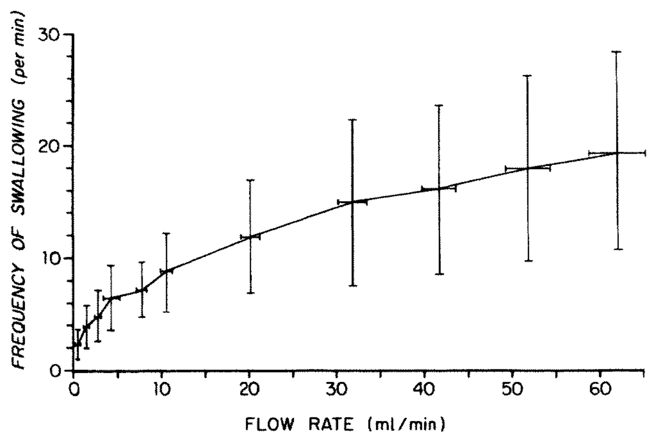

Fig. 2 – The effect of fluid infusion rate on the frequency of swallowing. The vertical lines indicate standard deviations for the swallowing frequency, while the horizontal lines indicate standard deviations for the fluid flow rate.

amount of acid production by the bacteria in the dental plaque. Therefore, individual variations in the values of RESID and VMAX could be of importance as determinants of susceptibility to dental caries.

Some methodological problems of measuring the residual volume need to be pointed out. First, the borders of the oral cavity are not well-defined. It was assumed that the saliva volume mixing with water when rinsing coincided with the volume important for clearance. Second, we assumed for our dilution technique that the ions used as markers were evenly distributed and did not bind to the mucosa. Preliminary studies using magnesium as a marker gave spurious results because of the tendency of the magnesium ion to bind to the oral mucosa. If potassium had a slight tendency to bind to the oral mucosa, this could perhaps explain the slightly higher value for RESID using chloride as a marker as compared with potassium.

In the present study, the residual volume was found to vary to a high degree between subjects, whereas the within-subject variation was much less. Inter-individual variation could be partly due to such factors as different rinsing techniques, variations in salivary viscosity, or the morphology of the teeth. The volume of saliva retained in the mouth could be dependent on the size of the interproximal spaces between the teeth. These spaces tend to enlarge with increasing age and in pathological conditions such as periodontal disease.

The differences in RESID between the sexes could perhaps be explained by differences in height and weight if these are correlated with the size of the oral cavity, although in each sex, height and weight were not correlated with RESID.

A rather surprising finding was the small difference in RESID following normal and forced swallows. The results imply that, even with a normal swallow, the volume of saliva remaining in the mouth is close to the lower limit and could not be much reduced by forced swallowing. The value of RESID after a normal swallow (0.77 ml) was considerably higher than that of 0.3 ml assumed by Dawes (1983) in development of the computer model of salivary clearance and the range of 0.3-0.6 ml determined by Dodds & Edgar (1983) by an unstated method.

In a preliminary study, the mucosal surface areas of the oral cavities of three subjects were measured by cutting out filter paper to cover all surfaces as far back as the fauces, including the tongue. The areas ranged from 200 to 250 cm^2. This indicates that, if the mouth is closed, and if the residual volume of saliva is evenly distributed and the mucosal surfaces are in contact with each other, then the salivary layer separating adjacent mucosal surfaces is only about 60 μm thick. This layer would increase to approximately 100 μm before the next swallow. This thin layer of saliva makes it likely that the equilibrium of ions and molecules between the saliva and the dental plaque or the surfaces of the teeth should be rapidly established.

The volume of the salivary bolus normally swallowed does not seem to have been studied. Lear *et al.* (1965) found the mean swallowing frequency to be one swallow each 98.6 sec in subjects sitting reading, from which an approximate volume swallowed of 0.5 ml can be calculated, assuming that their subjects had the normal salivary flow rate of 0.32 ml/min reported by Becks and Wainwright (1943). In the present study, the mean swallowing frequency was much higher – one swallow each 26 sec – and the mean resting salivary flow rate was also somewhat higher (0.50 ml/min) than the mean value given by Becks and Wainwright (1943), although the range which they reported encompasses all our results. Very recently, Calloway *et al.* (1982) reported a much lower swallowing frequency (one swallow/333 sec) in a group of normal subjects.

There is no obvious explanation for these differences, since the methods of recording swallowing were very similar, and psychic effects would be expected to be similar in all three studies. Both Lear *et al.* (1965) and Calloway *et al.* (1982) used either a strain gauge or microphone taped over the larynx, and our own equipment appears very similar to that illustrated in Fig. 3 of the article by Lear *et al.* (1965). It is difficult to assess whether the recording procedure influenced the rate of swallowing, particularly at the unstimulated flow rate. However, in the results shown in Fig. 1, in which the volume swallowed shows a relationship to the rate of fluid infusion into the oral cavity, there is no discontinuity in the curve at the unstimulated salivary flow

rate. Furthermore, the subjects were not informed of the purpose of the swallowing records.

Although it is not known whether RESID increased during fluid infusion, Fig. 1 shows that the volume swallowed (and thus VMAX) increased with fluid infusion rate. It is uncertain whether an exactly similar relationship would have been followed for stimulated saliva rather than water, since McNamara and Moyers (1973) reported somewhat different electromyographic patterns in monkeys for the oral phase of swallowing of saliva and water. Nevertheless, the computer model of oral sugar clearance (Dawes, 1983) was altered to make VMAX dependent on the salivary flow rate. The relationship used was

$$\mathrm{VMAX} = \mathrm{RESID} + 0.198 + 0.183\ (\mathrm{FR}) - 0.0063\ (\mathrm{FR})^2$$

which was derived by a least-squares fit of a parabola to the data in Fig. 1 (multiple $R = 0.9979$). Computer simulation with this altered model showed that variation of VMAX with salivary flow rate had only minor effects on the rate of sugar clearance.

An interesting finding was that, when flow of fluid into the mouth increased, there was an increase in both the volume swallowed (Fig. 1) and the frequency of swallowing (Fig. 2). It is uncertain whether there are receptors which monitor the volume or rate of entry of fluid or saliva into the mouth. Indeed, the afferent inputs controlling the rate of swallowing and the volume swallowed are probably receptors in the laryngeal area (Storey, 1968).

The volume swallowed, with each swallow at the highest rate of fluid flow into the mouth (62.0 ml/min), averaged about 4 ml. This is still much less than the value of about 17 ml which has been reported when adults are drinking a glass of water (Jones and Work, 1961). The reason for this discrepancy may be that the water infusion triggers a laryngeal protective reflex, whereas the water drinking is an alimentary supportive reflex dependent on pharyngeal receptors (Storey, 1976).

The main objective of the study was to determine normal values for RESID and VMAX, since these are predicted to have marked effects on the rate of sugar clearance and thus possibly on dental caries. Our subjects were not suitable for assessing the relationship between the studied variables and the prevalence or incidence of dental caries. However, the fact that in males we found a weak positive correlation ($p < 0.05$) between the caries prevalence and the residual volume after swallowing indicates that further studies of these salivary parameters are worth pursuing.

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